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**Evaluation of the use of non-pathogenic porcine circovirus type 1 as a vaccine delivery
virus vector to express antigenic epitopes of porcine reproductive and respiratory
syndrome virus**

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Running title: PCV1 as a vaccine delivery virus vector

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Abstract

We previously demonstrated that the C-terminus of the capsid gene of porcine circovirus type 2 (PCV2) is an immune reactive epitope displayed on the surface of virions. Insertion of foreign epitope tags in the C-terminus produced infectious virions that elicited humoral immune responses against both PCV2 capsid and the inserted epitope tags, whereas mutation in the N terminus impaired viral replication. Since the non-pathogenic porcine circovirus type 1 (PCV1) shares similar genomic organization and significant sequence identity with pathogenic PCV2, in this study we evaluated whether PCV1 can serve as a vaccine delivery virus vector. Four different antigenic determinants of porcine reproductive and respiratory syndrome virus (PRRSV) were inserted in the C-terminus of the PCV1 capsid gene, the infectivity and immunogenicity of the resulting viruses are determined. We showed that an insertion of 12 (PRRSV-GP2 epitope II, PRRSV-GP3 epitope I, and PRRSV-GP5 epitope I), and 14 (PRRSV-GP5 epitope IV) amino acid residues did not affect PCV1 replication. We successfully rescued and characterized four chimeric PCV1 viruses expressing PRRSV linear antigenic determinants (GP2 epitope II: aa 40–51, ASPSHVGWWSFA; GP3 epitope I: aa 61–72, QAAAEAYEPGRS; GP5 epitope I: aa 35–46, SSSNLQLIYNLT; and GP5 epitope IV: aa 187–200, TPVTRVSAEQWGRP). We demonstrated that all chimeric viruses were stable and infectious *in vitro* and three chimeric viruses were infectious *in vivo*. An immunogenicity study in pigs revealed that PCV1-VR2385_{EPI} chimeric viruses elicited neutralizing antibodies against PRRSV-VR2385. The results have important implications for further evaluating PCV1 as a potential vaccine delivery vector.

Keywords: Vaccine delivery vector; Porcine circovirus type 1 (PCV1); Porcine reproductive and respiratory syndrome virus (PRRSV); Antigenic epitopes; Porcine circovirus type 2 (PCV2)

1. Introduction

Porcine circoviruses (PCV) belong to the genus *Circovirus* of the family *Circoviridae* (Finsterbusch and Mankertz, 2009). The viral genome is packaged in an icosahedral capsid approximately 17 nm in diameter, and PCV is the smallest virus infecting mammals. Two types of PCV, PCV1 and PCV2, have been identified thus far. PCV1 was first described in 1974 as a contaminant of the porcine kidney cell line, PK-15, and is non-pathogenic in pigs (Tischer et al., 1982). PCV2 is pathogenic and causes an economically-important porcine circovirus-associated diseases (PCVAD) in swine worldwide (Allan et al., 1998; Allan et al., 1999; Ellis et al., 1998). Both PCV1 and PCV2 are non-enveloped, single-stranded circular DNA molecules of 1,759 (PCV1) and 1,768 (PCV2) kb in size (Finsterbusch and Mankertz, 2009).

The non-pathogenic PCV1 shares similar genomic organization with the PCVAD-associated PCV2 (Chae, 2005), which is characterized by 11 potential open reading frames (ORFs) with predicted protein sizes ranging from 2 to 36 kDa (Hamel et al., 1998). However, thus far only two major ORFs are believed to be essential for completing the basic functions of the virus: ORF1 encodes the replicase (Rep) (314 aa) and the truncated, spliced Rep' (178 aa), whereas the ORF2 encodes the immunogenic capsid protein (233 aa). Sequence analyses revealed that PCV1 shares a 76% nucleotide sequence identity with its pathogenic counterpart PCV2. The ORF1-encoded replicase protein has approximately 80% amino acid sequence identity between the two viruses, whereas the ORF2 capsid protein has about 60% amino acid sequence identity (Mahé et al., 2000; Tribble and Rowland, 2012). The ORF1 and ORF2 genes are oriented in opposite directions, resulting in an ambisense orientation. Between the 5' end of ORF1 and ORF2, there exists an intergenic region that contains the origin of virus replication characterized by a stem-loop structure (Mankertz et al., 2004).

Since the initial identification of PCV2 (Ellis et al., 1998), several genotypes have now been described (Jantafong et al., 2011; Wang et al., 2009) and demonstrated to co-exist in pigs (Allan et al., 2012; Zhai et al., 2011). For a single-stranded DNA virus, PCV2 has been shown to have the highest DNA mutation rate that is comparable to single-stranded RNA viruses (Firth et al., 2009). In contrast, the non-pathogenic PCV1 has been demonstrated to have a low mutation rate and low genetic diversity worldwide (Cortey and Segalés, 2012; Tombácz et al., 2014). While PCV2 is highly prevalent in most swine-producing countries and is associated with clinical PCVAD, PCV1 is non-pathogenic and has a low prevalence in swine herds (Allan et al., 1994; Allan et al., 1995; Calsamiglia et al., 2002; Dulac and Afshar, 1989; Edwards and Sands, 1994; Kim and Chae, 2001; Krakowka et al., 2000; Magar et al., 2000; Tischer et al., 1986). A recent survey demonstrated that, while PCV2 DNA and PCV2-specific antibodies are present in more than 80% of the samples evaluated, the molecular and serological prevalence of PCV1 is less than 2.4% (Puvanendiran et al., 2011).

We previously demonstrated that a genetically modified infectious PCV2 can tolerate up to a 27 aa insertion in the C-terminus of the ORF2 capsid gene (Beach et al., 2011). We showed that insertion of single, dimeric, and trimeric hemagglutinin (HA) tags, a GLu-GLu epitope tag of a mouse polyomavirus, and the KT3 epitope tag of the simian virus 40 in the C-terminus of PCV2 capsid gene resulted in infectious chimeric viruses that induce both PCV2-neutralizing antibodies and anti-epitope tag antibodies (Beach et al., 2011). Another study reported that insertion of a VP1 epitope region (aa 141–160, LTNVRGDLQVLAQKAARPLP) of the foot and mouth disease virus (FMDV) in PCV2 produced infectious virus *in vitro* and in a mouse model, and the PCV2-FMDV chimera elicited dual immunity against PCV2 and FMDV (Huang et al., 2014). We recently demonstrated that chimeric PCV1-2a vaccine can tolerate the insertion of

linear PRRSV epitope and induce dual immunity again PCV2 and PRRSV as a potential bivalent vaccine (Pineyro et al., 2015)

Because of the low prevalence of PCV1 in swine herds, the non-pathogenic nature, the low mutation rate, and the systemic tropisms of PCV1 for multiple tissues and organs, it is logical to explore the potential use of PCV1 as a vaccine delivery virus vector. Therefore, in this study, as a proof-of-principle, we evaluated whether PCV1 can express known antigenic determinants of porcine reproductive and respiratory syndrome virus (PRRSV), an economically-important swine pathogen. Generation of chimeric viruses containing neutralizing antigenic epitopes of PRRSV in the backbone of the non-pathogenic PCV1 could potentially elicit protective immunity against PRRSV with the benefit of a live virus-vectored vaccine, but without the risk of pathogenicity or reversion to virulence often associated with the traditional modified live-attenuated vaccines.

2. Materials and methods

2.1. Construction of chimeric PCV1-PRRSV_{EPI} (epitope) infectious clones:

Four different known antigenic epitopes derived from PRRSV strain VR2385, including GP2 epitope II (aa 40–51, ASPSHVGWWSFA), GP3 epitope I (aa 61–72, QAAAEAYEPGRS), GP5 epitope I (aa 35–46, SSSNLQLIYNLT), and GP5 epitope IV (aa 187–200, TPVTRVSAEQWGRP), were each cloned individually in frame into the C-terminus of the PCV1 capsid gene (GenBank accession number GU799575). Chimeric viruses were constructed by overlapping extension and fusion PCR following a method previously described (Beach et al., 2011). Briefly, a pCR2.1-PCV1 infectious clone plasmid containing the full-length PCV1 genome was used as the template to generate two amplicons of 200 bp and 1,800 bp with

complementary overhangs containing individual PRRSV antigenic epitope sequences (**Table 1**). A second round of fusion PCR was performed to assemble the previously synthesized amplicons. The PCR product was digested with *KpnI* and inserted into the pCR2.1TOPO vector (Invitrogen) (**Fig. 1**). Recombinant plasmids containing the insert were transformed into the alpha-select strain of *E. coli* (Bioline). Positive clones were selected, and insertion of each specific PRRSV epitope was confirmed by DNA sequencing. The viral genomic DNA was excised from the plasmid by enzymatic digestion with *KpnI* and concatemerization was carried out through a ligation reaction with T4 DNA ligase (Invitrogen), overnight, at room temperature. The infectious chimeric virus was generated by transfection of the concatemerized genomic DNA into the PCV1-free PK15 cells at 30%–40% confluency with lipofectamine ltx (Invitrogen). After 72 h post-transfection, the infectious virus was harvested by three cycles of freezing and thawing of the cells.

2.2. *In vitro* infectivity, epitope expression, and titration of chimeric PCV1-PRRSV_{EPI} viruses:

PCV1-free PK-15 cells were seeded at a concentration of 2×10^5 cells/well in a 48-well plate. After reaching approximately 40%–50% confluency, cells were washed once with Hank's Balanced Salt Solution (Gibco). The cells were then incubated with 100 μ L of 1:10 serial dilution of the virus stock for 1 h at 37°C in 5% CO₂, after which the cells were washed once with 200 μ L of minimum essential media (MEM) (Gibco). Infection was carried out in 300 μ L MEM supplemented with 10% FBS (Invitrogen) and 1% antibiotic/antimycotic (Fisher) at 37°C in 5% CO₂. After 72 h post-infection, the cells were fixed with 80% acetone and the infectivity was assessed by an immunofluorescence assay (IFA).

Briefly, infected cells were incubated with 100 μ L of mouse anti-PCV1-Cap monoclonal antibody, followed by fluorescein isothiocyanate-conjugated goat anti-mouse IgG (KPL, Kirkegaard & Perry Laboratories, Inc.). Expression of PRRSV-specific antigenic epitopes was also confirmed by IFA using custom polyclonal rabbit antibodies (Biomatik) against each synthetic PRRSV epitope followed by secondary goat anti-rabbit IgG (DyLight 550). Cells positive for both PCV1 Cap and PRRSV epitopes were visualized using a Zeiss LSM 880 confocal microscope (Zeiss, Pleasanton, CA). Serial ten-fold dilutions of the virus stock were performed in order to determine the 50% tissue culture infectious dose (TCID₅₀) of the virus stocks according to the method described by of Reed and Muench (Reed and Muench, 1938).

2.3. *In vivo* characterization of the infectivity and immunogenicity of four PCV1-PRRSV_{EPI} chimeric viruses

2.3.1. Experimental design for the animal study

A total of 21 5-weeks-old specific-pathogen-free (SPF) pigs were randomly assigned into seven groups of three pigs each, including two positive control groups (PCV1 and PRRSV), a negative control (MEM-treated group), and four groups for each of the PCV1-PRRSV_{EPI} chimeric viruses. Pigs in each of the PCV1-PRRSV_{EPI} chimeric virus groups and the PCV1-positive control group were intramuscularly inoculated with 5 mL (4.64×10^2 TCID₅₀/mL) of the respective viruses. Pigs in the PRRSV-VR2385-positive control group were each inoculated with 5 mL (2×10^5 TCID₅₀/mL) of PRRSV-VR2385. Serum samples were collected from each pig prior to inoculation and weekly thereafter for a period of 7 weeks.

2.3.2. Quantification of viral DNA loads in sera and lung tissues

The viral DNA was extracted from serum samples at 0, 7, 14, 21, 28, 35, and 42 days post-inoculation (dpi) and from tissues (lung and tracheobronchial lymph node) at 42 dpi, using Ambion MagMAX-96 Viral DNA Isolation kit (Thermo Fisher Scientific) according to the manufacturer's instructions. The DNA standards used for the qPCR were virus stock used for inoculation as well as plasmids containing the full-length PCV1 infectious clone. In order to rule out potential cross-contamination, DNA extracted from PCV2a and PCV2b virus stocks, and empty pCR2.1TOPO vector (Invitrogen), were included as the negative control. The PCV1 DNA copy numbers in sera or tissues were quantified by a TaqMan[®] Fast Virus 1-Step Master Mix (Life Technologies Corp.) according to the manufacturer's protocol. The PCR primers and probes (PCV1 P9/PCV1 P10/PCV1 probe) (**Table 1**) used in the qPCR assay were designed to target a specific amplicon of 97 bp in ORF2. The qPCR assay was conducted using the ABI 7500 (RT) PCR system (Life Technologies Corp). The PCR conditions included denaturation at 95°C for 20 s, annealing at 95°C for 3 s, amplification at 60°C for 30 s, and a final extension at 72°C for 5 min, with a total number of 40 cycles. Each reaction was performed in triplicate.

2.3.3. Serological evaluation of anti-PCV1 antibodies and anti-PRRS_{EPI} antibodies:

Specific antibodies against PCV1-Cap were tested by an indirect immunofluorescence assay (IIFA). For the IIFA, the PCV1-free PK-15 cells were inoculated with 100 µL of PCV1 and incubated for 72 h at 37°C, and fixed in 80% acetone. Sera from pigs in each of the chimeric PCV1-PRRSV_{EPI}-infected groups as well as from pigs in both the positive and negative control groups were serially diluted and incubated for 1 h at 37°C. Cells were washed three times with PBS, followed by addition of 100 µL (1:100) of fluorescent-labeled secondary anti-swine IgG

antibody (KPL, Kirkegaard & Perry Laboratories, Inc.). The cells were then washed again, and positive cells were detected using a fluorescence microscope. The virus titer was defined as the highest positive dilution and expressed as a mean geometric titer. The anti-PRRSV N antibody response was also evaluated using the IDEXX HerdCheck X3 ELISA kit according to the manufacturer's instructions.

Four different PRRSV KLH-conjugated synthetic antigenic peptides (GP2 epitope II: aa 40–51, ASPSHVGWWSFA; GP3 epitope I: aa 61–72, QAAAEAYEPGRS; GP5 epitope I: aa 35–46, SSSNLQLIYNLT; and GP5 epitope IV: aa 187–200, TPVTRVSAEQWGRP) were used as the antigen for the four PRRSV peptide-based antibody ELISAs. Each vial of lyophilized peptide (5 mg) was resuspended in 1 mL of UltraPure™ Distilled Water (Gibco®, Life Technologies) to a final stock concentration of 5 mg/mL, aliquotted, and stored at –80°C. Following titration and optimal dilution, 96-well microtitration plates (Nunc, Thermo Fisher Scientific) were manually coated with 100 µL per well of each peptide at a concentration of 5 µg/mL in phosphate-buffered saline (PBS) at pH 7.4 (Gibco®, Life Technologies) and incubated at 4°C overnight. The ELISA conditions, including coating and blocking, buffers, sample and conjugate dilutions, and incubation conditions (time and temperature), were identical for the four different peptide-based ELISAs. Serum samples were diluted at 1:50, after which plates were loaded with 100 µL of the diluted sample per well. Plates were incubated at 37°C for 1 h and washed five times with PBS containing 0.1% Tween 20. Subsequently, 100 µL of peroxidase-conjugated goat anti-pig IgG (Fc) antibody (Bethyl Laboratories Inc.), diluted at 1:15,000, were added to each well and the plates were incubated at 37°C for 1 h. After a washing step, the reaction was visualized by adding 100 µL of tetramethylbenzidine-hydrogen peroxide (Dako North America, Inc.) substrate solution to each well. After 10 min incubation at room

temperature, the reaction was stopped by the addition of 50 μ L of a stop solution (1 M sulfuric acid) to each well. Reactions were measured according to the optical density at 450 nm using an ELISA plate reader (Biotek[®] Instruments Inc.) operated with commercial software (GEN5TM, Biotek[®] Instruments Inc.).

2.3.4. Serum virus neutralization assay to evaluate the neutralizing activity against PRRSV VR2385:

The neutralizing antibody titers against PRRSV-VR2385 were determined by a serum virus neutralization assay essentially as previously described (Zhou et al., 2012). Briefly, two-fold diluted serum samples collected at 28, 35, and 42 dpi from each pig were mixed with an equal volume of the PRRSV VR2385 virus at an infectious titer of 2×10^3 TCID₅₀/mL and incubated at 37°C for 1 h. The mixtures were then inoculated onto MARC-145 cells in 96-well plates and incubated for 1 h at 37°C. After washing with PBS, the cells were maintained in DMEM with 2% FBS. At approximately 20 hpi, the cells were assayed by IFA for virus infection. The neutralizing antibody titers were expressed as the highest dilution that showed a 90% or above reduction in the number of fluorescent foci compared to that of antisera from negative control pigs. Samples were evaluated in triplicate and three independent tests were performed for each serum sample.

2.4. Statistical analysis

The Student's *t*-test (unpaired) was used to evaluate the differences ($P < 0.05$) between the samples in the two groups. Repeated measure two-way ANOVA with Tukey's correction was calculated for multiple comparison. Statistical significance was set to $\alpha = 0.05$. All analyses

were performed using commercially available software GraphPad Prism® 6 (GraphPad Software, Inc, CA).

3. Results

3.1. Chimeric PCV1 viruses containing PRRSV VR2385 antigenic epitopes inserted in the C-terminus of the PCV1 capsid are infectious *in vitro*:

Each of the chimeric PCV1-PRRS_{EPI} clones was verified by full-length genomic sequencing for the presence in frame of each of the inserted PRRSV antigenic epitopes in the C-terminus of the PCV1 capsid gene. Transfection of each full-length chimeric virus DNA clone in PK-15 cells resulted in the production of infectious virions. Confocal microscopy revealed that each of the PCV1-PRRS_{EPI} chimeric viruses expressed PCV1 Cap as well as the respective PRRSV antigenic determinant (**Fig. 2**). Infected cells showed dual nuclear staining with both anti-PCV1 monoclonal antibodies and anti-PRRSV epitope peptides (GP2 II, GP3I, GP5I, and GP5IV) mono-specific antibodies. There was no significant difference in the genomic copy numbers between wild-type PCV1 and PCV1-PRRS_{EPI} chimeric viruses at 96 hpi (data not shown). The stability of chimeric viruses was confirmed after five successful serial passages in PK-15 followed by dual IFA staining of the PCV1 capsid and respective PRRSV antigenic determinant, as well as by sequence confirmation of the chimeric viruses harvested after the five passages (data not shown).

3.2. PCV1-VR2385_{EPI} chimeric viruses are viremic and replicate in tissues of experimentally inoculated pigs:

All serum samples, evaluated by TaqMan® qPCR for the presence of PCV1 prior to inoculation at day 0, were negative. Viremia was detected at as early as 7 dpi for PCV1-

VR2385_{EPI}GP3I and PCV1-VR2385_{EPI}GP5IV viruses, and at 21 dpi for PCV1-VR2385_{EPI}GP2II and PCV1-VR2385_{EPI}GP5I viruses (**Table 2**). The frequency of pigs showing viremia in each group varied during the trial. The number of animals used and the frequency variability do not allow for robust statistical analysis; however, the average DNA viral loads in serum samples from each chimeric virus were within one log₁₀ difference: 1.39×10^5 genomic copies/mL for PCV1-VR2385_{EPI}GP2II; 1.87×10^5 genomic copies/mL for PCV1-VR2385_{EPI}GP3I; 4.22×10^5 genomic copies/mL for PCV1-VR2385_{EPI}GP5I; and 1.96×10^5 genomic copies/mL for PCV1-VR2385_{EPI}GP5IV, and had at least two-log₁₀ lower genomic copies/mL than the parental PCV1 (1.26×10^7 genomic copies/mL) (**Fig. 3A**). After 42 dpi, all animals were necropsied and no significant pathological lesions were observed. However, all infected groups had detectable viral DNA in the tracheobronchial lymph nodes and lungs (**Table 2**), indicating virus replication in tissues. No significant differences in viral genomic copy number/gram of tissue were observed between parental PCV1 and PCV1-VR2385_{EPI} chimeric viruses in tracheobronchial lymph nodes and lungs (**Fig. 3B**). No evidence of PCV1 replication was observed in the PRRSV-VR2385 and MEM control groups.

3.3. PCV1-VR2385_{EPI} chimeric viruses induce both PCV1-specific and PRRSV antigenic epitope-specific antibodies in pigs:

Anti-PCV1 IgG antibodies were detected in the sera of the wild-type PCV1 control group as well as all the PCV1-VR2385_{EPI} chimeric viruses-inoculated groups. IgG anti-PCV1 antibodies were detected from 14 dpi in wild-type PCV1-infected pigs and remained seropositive at 42 dpi. Anti-PCV1 IgG antibodies were detected in PCV1-VR2385_{EPI}GP3I- and PCV1-VR2385_{EPI}GP5IV-infected groups at 14, 21, and 28 dpi, followed by a significant titer reduction

compared to the wild-type PCV1 at 35 and 42 dpi. The remaining chimeric viruses-infected groups, PCV1-VR2385_{EPI}GP2II and PCV1-VR2385_{EPI}GP5I, showed a delayed seroconversion to anti-PCV1 IgG antibodies (35 dpi) and significantly lower levels of anti-PCV1 IgG antibodies, compared to the wild-type PCV1-infected group, at 35 and 42 dpi (**Fig. 4A**). Anti-PCV1 IgG antibodies were not detected in PRRSV or MEM control groups. As expected, anti-PRRSV N antibodies were only detected in the PRRSV 2385 infected group (**Fig. 4B**).

Antibody responses against the inserted PRRSV antigenic epitopes were detected by specific epitope-based ELISA. Antibody response against PRRSV-GP2 epitope II was not detected in pigs experimentally infected with the PCV1-VR2385_{EPI}GP2II chimeric virus (**Fig. 5A**). However, specific antibodies against PRRSV-GP3 epitope I were detected in the PCV1-VR2385_{EPI}GP3I chimeric virus group at 28 dpi and remained positive at 42 dpi (**Fig. 5B**). Specific antibodies against PRRSV-GP5 epitope I were detected at 21 dpi in the wild-type PRRSV VR2385-infected group, and at 35 dpi in the PCV1-VR2385_{EPI}GP5I chimeric virus-infected group at 35 dpi, and remained positive at 42 dpi (**Fig. 5C**). The presence of anti-PRRSV-GP5 epitope IV antibodies was detected at 21 dpi in the PCV1-VR2385_{EPI}GP5IV chimeric virus group and at 28 dpi in wild-type PRRSV VR2385 group, and remained at a high level in the PCV1-VR2385_{EPI}GP5IV chimeric virus group at 35 and 42 dpi (**Fig. 5D**). The low number of animals per group, as well as individual variation amongst animals may play a role in the different levels of antibodies response observed amongst groups.

3.4. PCV1-PRRSV_{EPI} chimeric viruses-infected pigs develop neutralizing antibodies against the PRRSV VR2385:

To investigate whether PCV1-VR2385_{EPI} chimeric viruses can induce neutralizing antibodies against PRRSV, a serum virus neutralization assay against PRRSV VR2385 strain was performed. Anti-PRRSV-VR2385 neutralizing antibodies were detected in the PCV1-VR2385_{EPI}GP3I, PCV1-VR2385_{EPI}GP5I, and PCV1-VR2385_{EPI}GP5IV chimeric viruses-infected groups at 28 dpi and remained detectable at 42 dpi. No statistical difference in neutralizing antibody titers were observed throughout the experiment between wild-type PRRSV VR2385 and PCV1-VR2385_{EPI}GP3I, PCV1-VR2385_{EPI}GP5I, and PCV1-VR2385_{EPI}GP5IV, except for PCV1-VR2385_{EPI}GP5I at 42 dpi (**Fig. 6**). PRRSV 2385 neutralizing antibodies were not detected in PCV1 or MEM control groups.

4. Discussion

PCV1 is a non-pathogenic virus in pigs infecting multiple tissues and organs (Allan et al., 1995; Calsamiglia et al., 2002; Krakowka et al., 2000). Early field studies reported a high serological prevalence of anti-PCV1 antibodies in the swine population (Dulac and Afshar, 1989; Edwards and Sands, 1994; Tischer et al., 1995), although no disease could be associated with the presence of this virus either naturally or experimentally (Allan et al., 1995; Tischer et al., 1986). However, more recent field studies have demonstrated that the serological prevalence of anti-PCV1 antibodies as well as virus circulation in the swine population are very low (Puvanendiran et al., 2011). Sequence and phylogenetic analyses have also demonstrated a low mutation rate and low genetic diversity of the PCV1 strains worldwide (Tomb  cz et al., 2014). Thus, the low prevalence of PCV1, lack of evidence of pathogenicity, low mutation rate, and systemic tropisms

for multiple tissues and organs make PCV1 an attractive candidate for a potential live vaccine vector.

Previous studies have successfully used the non-pathogenic PCV1 as the genomic backbone for the development of PCV2 vaccines (Fenaux et al., 2004). Cloning of PCV2 ORF2 capsid gene into the backbone of PCV1 proved to be viable *in vivo* and conferred full protection against PCV2, whilst still retaining the non-pathogenic nature of PCV1 (Fenaux et al., 2004; Fenaux et al., 2003). Additionally, pigs experimentally infected with a PCV2-1 reciprocal chimeric virus, containing PCV1 ORF2 in a PCV2 backbone, showed specific anti-PCV1 IgG antibody response (Fenaux et al., 2003). Herein in this study, we further demonstrated that insertion of known PRRSV antigenic determinants in the C-terminus of PCV1 ORF2 capsid gene produced infectious chimeric viruses and did not impair the humoral immune response against PCV1. Although different levels of anti-PCV1 IgG were detected, all PCV1-VR2385_{EPI} chimeric viruses were capable of induce anti-PCV1 IgG antibodies in infected SPF pigs.

The different levels of anti-PCV1 IgG antibodies might be associated with a different replication timeline and viral DNA load observed amongst the various PCV1-VR2385_{EPI} chimeric viruses compared with wild-type PCV1. The extension of the C-terminus of the PCV1 capsid gene through the addition of PRRSV antigenic epitopes might also affect the structural conformation and antibody induction capability of the PCV1 capsid. Indeed, previous studies demonstrated that mutation of the last four amino acid residues of the PCV2 ORF2 (-PLKP) to three amino acid residues of the PCV1 ORF2 (-LNK) reduces viral antibody recognition (Lekcharoensuk et al., 2004). Therefore, in the current study, anti-PCV1 IgG antibodies generated against PCV1-VR2385_{EPI} chimeric viruses may not completely bind to the full PCV1 Cap expressed by the wild-type PCV1 that was used for the IIFA serology test. Further studies

will be necessary to demonstrate whether the insertion of a foreign amino acid sequence at the C-terminus may alter the conformation of the PCV1 capsid protein.

It has been previously demonstrated that the C-terminus of the PCV2 capsid is a type-specific immune reactive epitope that is displayed on the surface of the virion capsid (Lekcharoensuk et al., 2004; Shang et al., 2009). In two separate studies, following the insertion of epitope tags in the C-terminal region, PCV2 and chimeric PCV1-2a vaccine were generated and shown to elicit dual immunity against both PCV2 capsid and the inserted epitopes, whereas mutation of the N-terminus of PCV2 capsid impaired viral replication (Beach et al., 2011; Pineyro et al., 2015). Herein in the present study, we inserted four different known PRRSV antigenic epitopes into the C-terminus of the non-pathogenic PCV1 capsid gene, and demonstrated that the insertions did not significantly affect virus infectivity *in vitro* or viral replication *in vivo*. We previously showed that epitopes as large as 27 amino acids can be inserted in the PCV2 capsid gene without impairing viral viability (Beach et al., 2011). In the present study, we demonstrated that insertions, varying from 12 aa for PRRSV-GP3 epitope I and PRRSV-GP5 epitope I, 12 aa for PRRSV-GP2 epitope II, and 14 aa for PRRSV-GP5 epitope IV, did not affect viral infectivity *in vitro* or replication *in vivo*. Future study is necessary to determine the tolerance of maximal length of amino acid insertion in PCV1 capsid without affecting the viability of the virus.

Although the PCV1 chimeric viruses generated in this study were capable of replicating *in vivo*, the rate of replication appears to be low. It has previously been demonstrated that PCV1 can replicate to a higher titer *in vitro*, compared to PCV2 (Beach et al., 2010a). However, to our knowledge, there is no information regarding the minimal infectious dose required for PCV1 infection *in vivo*. Previous studies using PCV1-2 chimeric virus in the backbone of PCV1

showed positive results with variable titers (Beach et al., 2010b; Fenaux et al., 2004; Pineyro et al., 2015). We therefore speculate that the low replication rate of the chimeric viruses observed in this study might be due to a low virus titer that was used for inoculation. Propagation of PCV1, PCV2 as well as chimeric viruses to higher infectious titers have been very challenging thus far.

In the current study, PCV1 chimeric viruses expressing four known B-cell linear epitopes of PRRSV, previously demonstrated to be immunogenic against PRRSV (de Lima et al., 2006; Plagemann, 2004b; Vanhee et al., 2011), were generated. It has been reported that GP5 plays a major role in PRRSV neutralization (Plagemann, 2004a; Plagemann, 2004b; Plagemann et al., 2002). PRRSV-GP5 epitope IV is an important immunogenic epitope (P¹⁸⁸LTR (V/T) SAEQW¹⁹⁷) that has also been proved to be reactive with sera raised against European PRRSV strains. Despite a few amino acid changes, this epitope seems to be well conserved amongst type 2 PRRSV strains (Oleksiewicz et al., 2001). We showed in this study that PCV1-VR2385_{EPI}GP5IV chimeric virus induced neutralizing antibody levels comparable to those induced by the PRRSV VR2385 virus. The PRRSV-GP5 epitope I neutralizing epitope, ³⁷SHLQLIYNL, for the PRRSV VR2332 is located in the GP5 ectodomain sequence and is considered as the primary neutralizing epitope for the type 2 PRRSV isolates (Plagemann, 2004b). The PCV1-VR2385_{EPI}GP5I chimeric virus in this study induced similar neutralizing antibody levels compared to those induced by PRRSV VR2385 virus at 28, and 35 dpi. However, the neutralizing antibody titers induced by the PCV1-PRRSV_{EPI} viruses appeared to decline more rapidly compared to the PRRSV VR2385, thus causing a significant reduction of neutralizing antibodies titer at 42 dpi.

PRRSV ORF3 is considered as the second most variable PRRSV structural protein, with four consecutive peptides from aa 61–105, all of which are considered as important immunodominant domains of GP3 (de Lima et al., 2006; Zhou et al., 2006). The PCV1-VR2385_{EPI}GP3I chimeric virus generated in this study contains the aa 61–72 (QAAAEAYEPGRS) and was shown to induce similar levels of neutralizing antibodies compared to the PRRSV VR2385. Despite the fact that PCV1-VR2385EPIGP2II chimeric virus is infectious *in vitro*, no viral DNA were detected in tissues of inoculated pigs, probably due to the short duration of viremia not being detectable with the current sampling scheme. This explanation was also supported by the presence of anti-PCV1 IgG at 14 dpi, indicative of virus replication. Furthermore, the chimeric virus also failed to induce antibodies against PRRSV-GP2 epitope II. Therefore, we have successfully demonstrated that three of the four PCV1-VR2385_{EPI} chimeric viruses generated in this study induced PRRSV epitope-specific antibodies and neutralizing antibodies against PRRSV VR2385 at a level comparable to those induced by wild-type PRRSV VR2385.

In summary, we successfully generated and rescued four PCV1 chimeric viruses expressing different known PRRSV linear-B epitopes (GP2 epitope II: aa 40–51, ASPSHVGWWSFA; GP3 epitope I: aa 61–72, QAAAEAYEPGRS; GP5 epitope I: aa 35–46, SSSNLQLIYNLT; and GP5 epitope IV: aa 187–200, TPVTRVSAEQWGRP). We further showed that three of these chimeric viruses were infectious *in vitro* and in pigs, and genetically stable. Importantly, we found that three PCV1-VR2385_{EPI} chimeric viruses elicited neutralizing antibodies against PRRSV-VR2385. Therefore, the results from the present study provided a proof of concept for further exploring the use of the non-pathogenic PCV1 as a live virus vector for vaccine delivery.

410

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418

419 **Conflict of interest statement:**

420 The corresponding author is the inventor of the chimeric PCV1-2a virus which is the basis for
421 the Zoetis Inc's Foster® PCV commercial vaccine. There is no other apparent conflict of
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567

Figure legends

Fig. 1. A schematic diagram for the construction of the PCV1-PRRSV_{EPI} chimeric DNA clones. The epitope insertion was accomplished by two rounds of overlapping extension PCR. The first amplicon of 200 bp containing an overhanging GPxx_{EPI} region (xx denote different inserted epitopes, GP2II, G3I, GP5I and GP5IV) was generated with M13-F and GPxx_{EPI}-R primers (black arrows). The second amplicon of 1778 bp containing a complementary GPxx_{EPI} overhanging region was generated with GPxx_{EPI}-F and M13-R primers (empty arrow heads). The full-length PCV1-PRRSV epitopes chimeric clones were assembled by a fusion PCR using previously generated amplicons as templates, and with M13-F and M13-R primers.

Fig. 2. Confocal microscopy of PK-15 cells infected with wild-type PCV1 as well as with four different PCV1-PRRSV_{EPI} chimeric viruses. PCV1-PRRSV_{EPI} chimeric viruses (PCV1-VR2385_{EPI}GP2II, PCV1-VR2385_{EPI}GP3I, PCV1-VR2385_{EPI}GP5I, and PCV1-VR2385_{EPI}GP5IV) and wild-type PCV1 were assayed by dual immunofluorescence staining. Infected cells were dually labeled with a mixture of mouse anti-PCV1 capsid monoclonal antibody (1:1000) (Mab) and PRRSV epitope-specific polyclonal antibodies (1:500) (Pab). In order to determine cross reactivity, cells infected with each specific chimeric virus group were tested against each respective PRRSV-specific epitope antibody. After incubation with the primary antibody, a mixture of fluorescein isothiocyanate (FITC) conjugated goat anti-mouse IgG (1:100) (KPL, Kirkegaard & Perry Laboratories, Inc.) and goat anti-rabbit IgG-DyLight (1:500) (Thermo Scientific) were added. Dually infected cells were visualized using a Zeiss LSM 880 confocal microscope (Zeiss, Pleasanton, CA) with a 40X objective, using the argon 488 and helium-neon 594 lasers.

Fig. 3. Detection and quantification of PCV1 viral DNA loads in serum, lymphoid tissues and lung samples in specific-pathogen-free (SPF) pigs experimentally infected with PCV1-PRRSV_{EPI} chimeric viruses. Pigs were experimentally infected with wild-type PCV1 as well as each of the four different PCV1-PRRSV_{EPI} chimeric viruses (PCV1-VR2385_{EPI}GP2II, PCV1-VR2385_{EPI}GP3I, PCV1-VR2385_{EPI}GP5I, and PCV1-VR2385_{EPI}GP5IV). Determination of viral DNA loads in serum and tissues was performed using TaqMan® qPCR. The number of animals used and the frequency variability in each time point, do not allow for robust statistical analysis. **(A)** Group mean log viral genomic copies/ml of serum is plotted for each treatment group, and the error bars indicate standard errors. **(B)** Mean viral DNA loads in tracheobronchial lymph node and lung were determined for each treatment group. Mean log viral genomic copies/gram of tissue is plotted for each treatment group, and the error bars indicate standard errors.

Fig. 4. Anti-PCV1 IgG antibodies and anti-PRRSV N antibodies in specific-pathogen-free (SPF) pigs experimentally infected with PCV1-PRRSV_{EPI} chimeric viruses. Pigs were infected with wild-type PCV1 as well as with each of the four different PCV1-PRRSV_{EPI} chimeric viruses. **(A)** Anti-PCV1 IgG antibodies were detected by an indirect immunofluorescence assay (IIFA). PCV1-free PK15 cells were infected with 1 MOI of wild-type PCV1. Immunoreactivity against PCV1 was evaluated in serum samples generated in pigs infected by wild-type PCV1, PCV1-PRRSV_{EPI} chimeric viruses, PRRSV-VR2385 and MEM control. Detectable anti-PCV1 IgG antibodies were seen as early as 14 dpi in wild-type PCV1, PCV1-VR2385_{EPI}GP3I, and PCV1-VR2385_{EPI}GP5IV, followed by PCV1-VR2385_{EPI}GP2II, and PCV1-VR2385_{EPI}GP5I at 21 dpi. Treatments with different letters represent statistically significant differences on that day. **(B)** The anti-PRRSV N antibody titers at indicated time

points were detected using the IDEXX HerdCheck X3 ELISA kit. The level of antibody was expressed as a sample/positive (S/P) value ratio. The dash line shows the cutoff threshold (S/P value ≥ 0.4). Each plot represents the mean value of 3 pigs per infected group at each time point. Statistical comparison was performed using repeated-measures analysis of variance, followed by Tukey's post-hoc procedure for multiple comparisons. Statistical significance was set to $\alpha = 0.05$.

Fig. 5. PRRSV antigenic epitope-specific ELISAs for detection of the inserted PRRSV epitope antibodies induced by PCV1-PRRSV_{EPI} chimeric viruses. Specific-pathogen-free pigs were infected with wild-type PCV1, as well as with each of the four PCV1-PRRSV_{EPI} chimeric viruses containing respective PRRSV antigenic epitopes. PRRSV epitope-specific antibody responses were tested in serum samples of pigs infected with PCV1-VR2385_{EPI}GP2II, PCV1-VR2385_{EPI}GP3I, PCV1-VR2385_{EPI}GP5I, and PCV1-VR2385_{EPI}GP5IV, wild-type PCV1, and PRRSV VR2385. All infected groups were tested individually against each epitope peptide. **(A)** PCV1-VR2385_{EPI}GP2II; **(B)** PCV1-VR2385_{EPI}GP3I; **(C)** PCV1-VR2385_{EPI}GP5I; and **(D)** PCV1-VR2385_{EPI}GP5IV. The average of three animals is plotted for each time point, and standard errors are indicated. Asterisks indicate significant differences on that day for each of the PCV1-PRRSV_{EPI} chimeric viruses compared to PRRSV VR2385 infected group. The dotted horizontal line indicates the cutoff of each assay. Statistical comparison was performed using two-way ANOVA followed Tukey's correction for multiple comparison. Statistical significance was set to $\alpha = 0.05$.

636 **Fig. 6. Kinetics of anti-PRRSV neutralizing antibody response in pigs experimentally**
 637 **infected with each of the four PCV1-VR2385_{EPI} chimeric viruses as well as with the PRRSV**
 638 **VR2385 virus.** Neutralizing antibody (NA) titers induced against the PRRSV VR2385 by each
 639 of the four PCV1-VR2385_{EPI} chimeric viruses as well as by the parental PRRSV VR2385 virus
 640 were detected as early as 28 dpi. NA antibodies titers observed in the PCV1-VR2385_{EPI}GP3I,
 641 PCV1-VR2385_{EPI}GP5I, and PCV1-VR2385_{EPI}GP5IV groups against PRRSV VR2385 were
 642 comparable to those observed in the PRRSV VR2385-infected group at 28 and 35 dpi. At 42
 643 dpi, the NA antibodies titers in PCV1-VR2385_{EPI}GP5IV group were significantly lower than
 644 those observed in the PRRSV-VR2385-infected group. The NA titers against parental strain
 645 PRRSV VR2385 and each of the PCV1-VR2385_{EPI} chimeric viruses were expressed as the
 646 highest dilution (2n) that showed a 90% or above reduction in the number of fluorescent foci
 647 compared to that of serum from negative control group. The NA titers against PCV1-VR2385_{EPI}
 648 GP2 II was not shown because of undetectable NA titer. Three independent experiments were
 649 performed for each test, and the error bars indicate standard errors. The P value shows whether
 650 one chimeric virus group had significant differences in NA titers compared to the parental
 651 PRRSV VR2385 group.

1 **Table 1. Primer sequences used in the construction and detection of the PCV1-PRRSV_{EPI} chimeric**
 2 **viruses**

Chimeric clone	Primer	Position	Primer sequence (5'-3')
PCV1-PRRSV _{EPI} GP2II	GP2II _{EPI} -P1	F47-59	CGCAAAGCTCCACCAGCCCACATGGCTCGGGCTCGCag ggtcttttagg
	GP2II _{EPI} -P2	R29-46	GCGAGCCCGAGCCATGTGGGCTGGTGGAGCTTTGCGcta aatgaataaaaaataa
PCV1-PRRSV _{EPI} GP3I	GP3I _{EPI} -P3	F47-59	GCTACGGCCCGGTTTCATACGCTTCCGCCGCCGCCTGagg gtcttttagg
	GP3I _{EPI} -P4	R29-46	CAGGCGGGCGGCGGAAGCGTATGAACCGGGCCGTAGCct aatgaataaaaaataa
PCV1-PRRSV _{EPI} GP5I	GP5I _{EPI} -P5	F47-59	GGTCAGGTTATAAATCAGCTGCAGGTTGCTGCTGCTagg gtcttttagg
	GP5I _{EPI} -P6	R29-46	AGCAGCAGCAACCTGCAGCTGATTTATAACCTGACCcta aatgaataaaaaataa
PCV1-PRRSV _{EPI} GP5IV	GP5IV _{EPI} -P7	F47-59	CGGACGGCCCCACTGTTCCGCGCTCACACGGGTCACC GGGGTtagggtcttttagg
	GP5IV _{EPI} -P8	R29-46	ACCCCGGTGACCCGTGTGAGCGCGGAACAGTGGGGCC GTCCG cttaatgaataaaaaataa
pCR2.1 TOPO	M13-R	R207-225	GGAAACAGCTATGACCATG
pCR2.1 TOPO	M13-F	F390-406	ACTGGCCGTCGTTTTAC
PCV1	P9	F505-525	CGATGTTGAATCTGAGGTGGT
PCV1	P10	R581-602	AGAAAGGCGGGAATTGAAGATA
PCV1	Probe	R528-553	ACATTCCAAGATGGCTGCGAGTATCC

Table 2. Detection of viremia and virus replication in tissues of pigs infected by parental PCV1, and each of the four PCV1-PRRSV_{EPI} chimeric viruses

Treatments	No. of pigs with detectable viremia and viral genome in tissues (positive/total no. of pigs)									
	dpi							Total	Lung	TBLN
	0	7	14	21	28	35	42			
PCV1-VR2385 _{EPI} GP2II	0/3	0/3	0/3	1/3	2/3	2/3	0/3	2/3	1/3	1/3
PCV1-VR2385 _{EPI} GP3 I	0/3	1/3	0/3	0/3	1/3	0/3	0/3	2/3	1/3	1/3
PCV1-VR2385 _{EPI} GP5 I	0/3	0/3	0/3	2/3	1/3	3/3	1/3	3/3	1/3	2/3
PCV1-VR2385 _{EPI} GP5IV	0/3	1/3	1/3	0/3	1/3	1/3	0/3	2/3	3/3	2/3
PCV1	0/3	2/3	1/3	1/3	1/3	1/3	2/3	2/3	2/3	2/3
PRRSV-VR2385	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3
Mock (MEM)	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3

6 dpi: days post infection, TBLN tracheobronchial lymph nodes

Fig 1

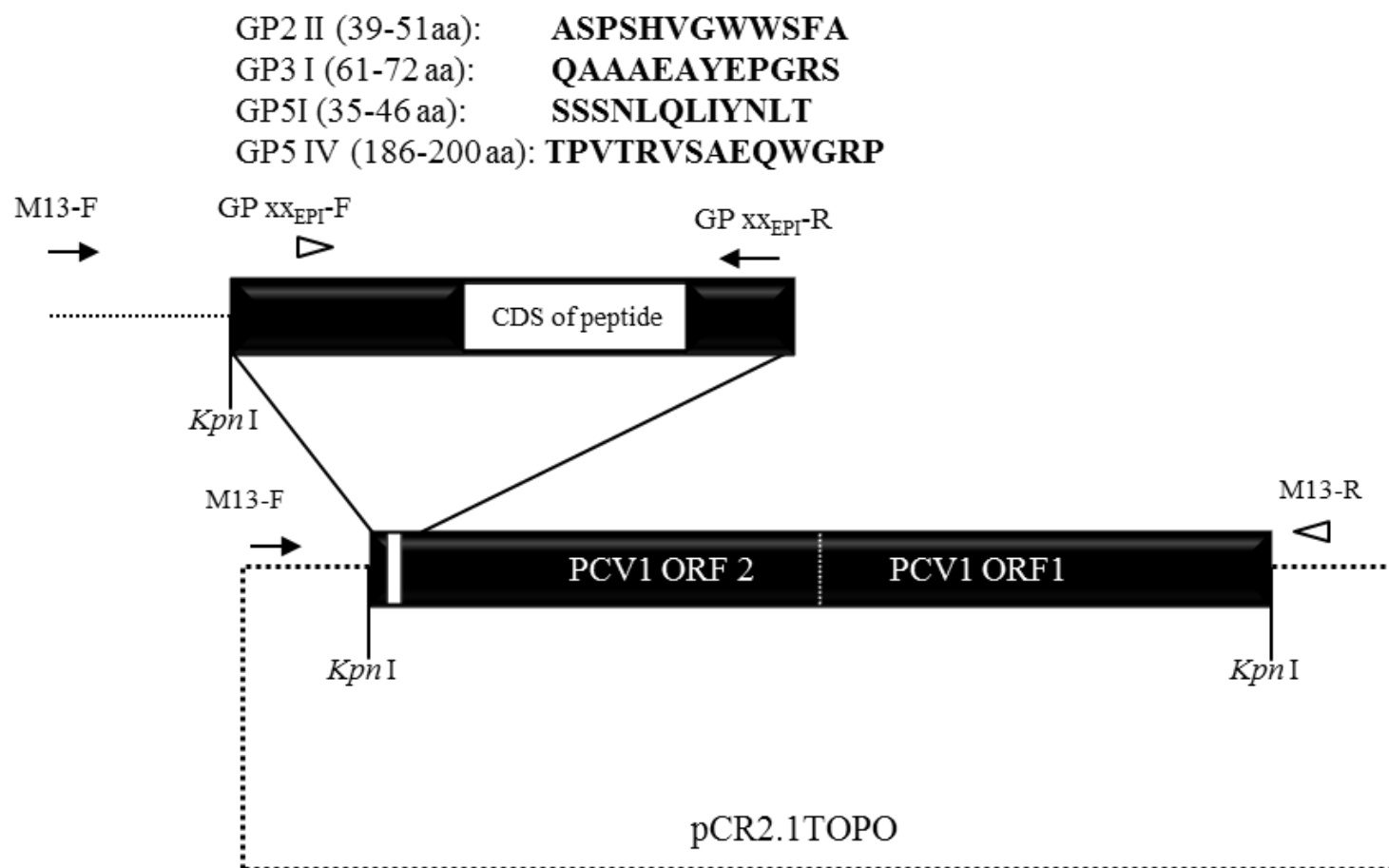


Fig 2

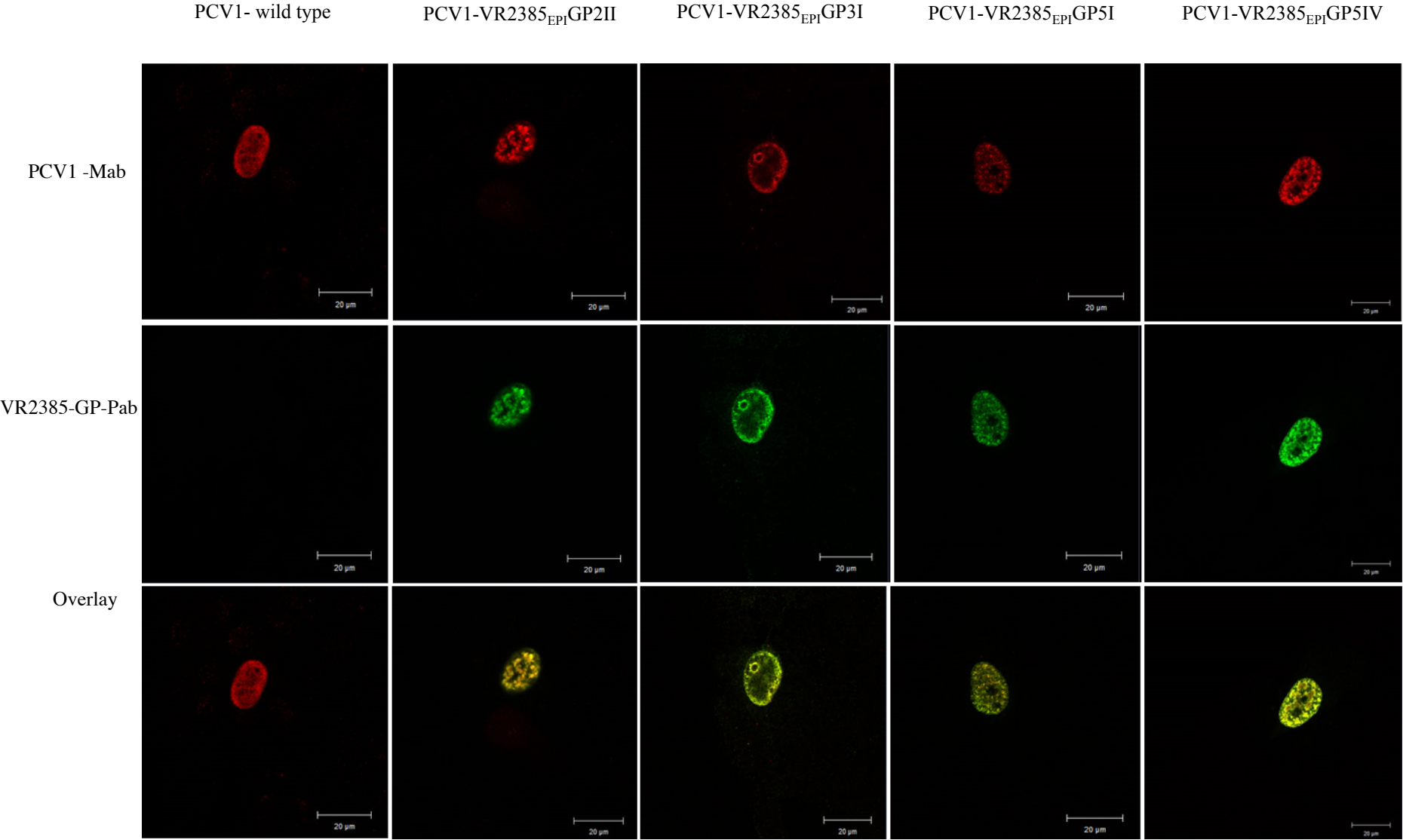


Fig 3

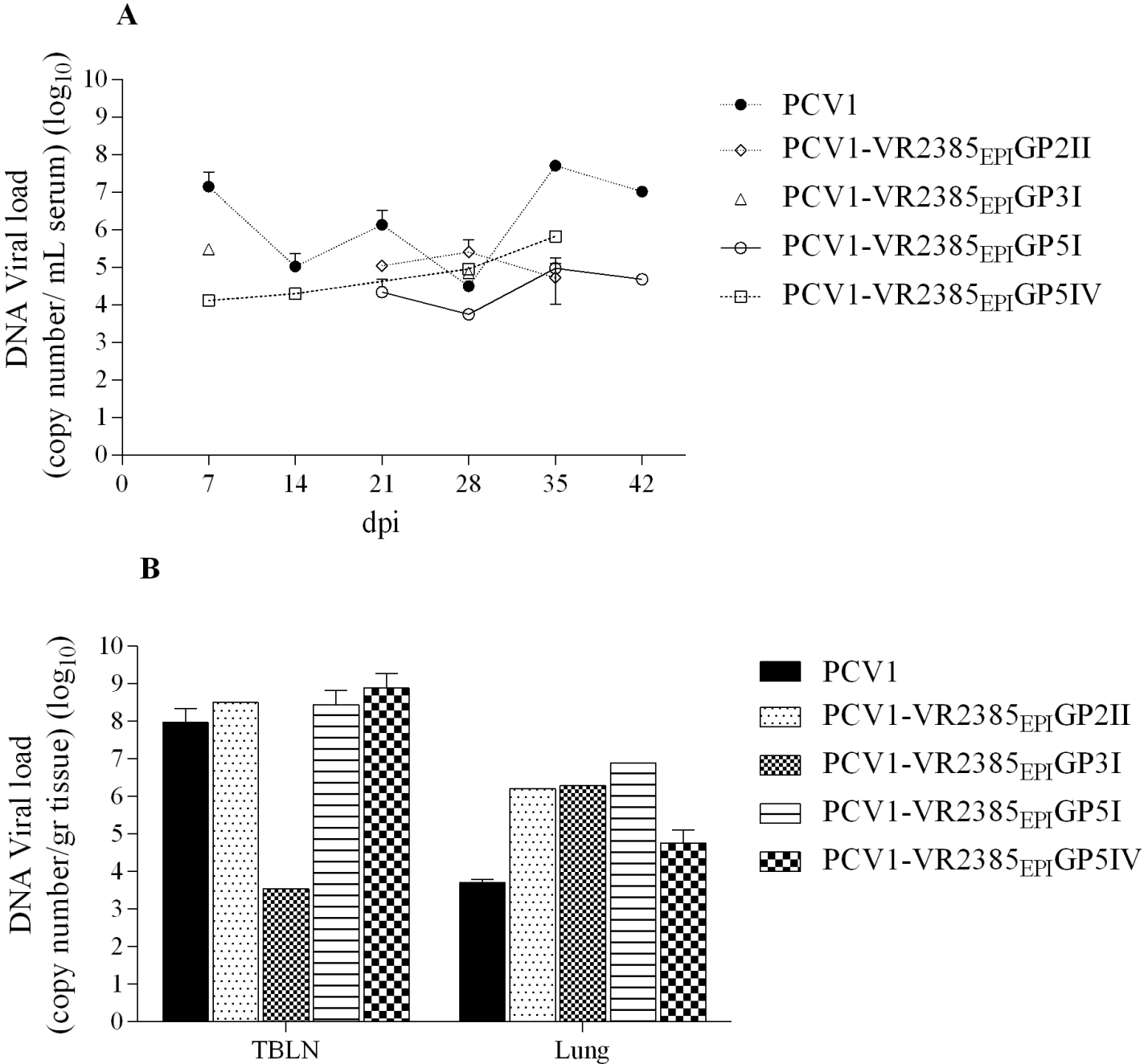


Fig 4

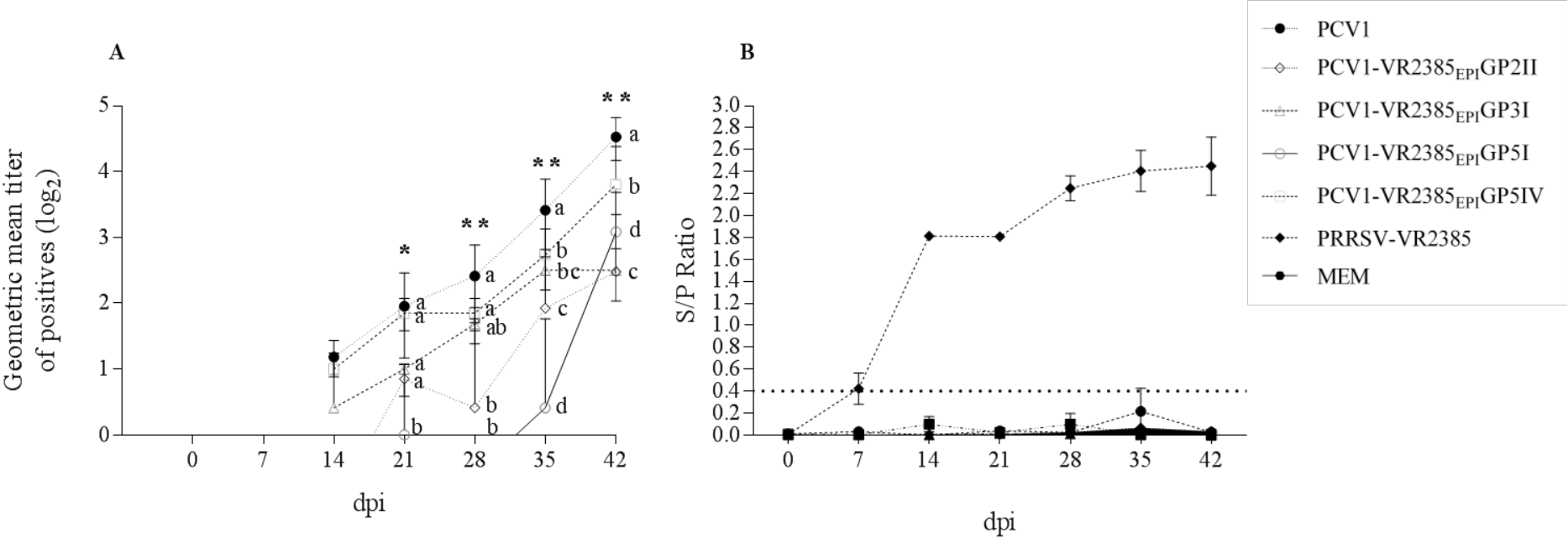


Fig 5

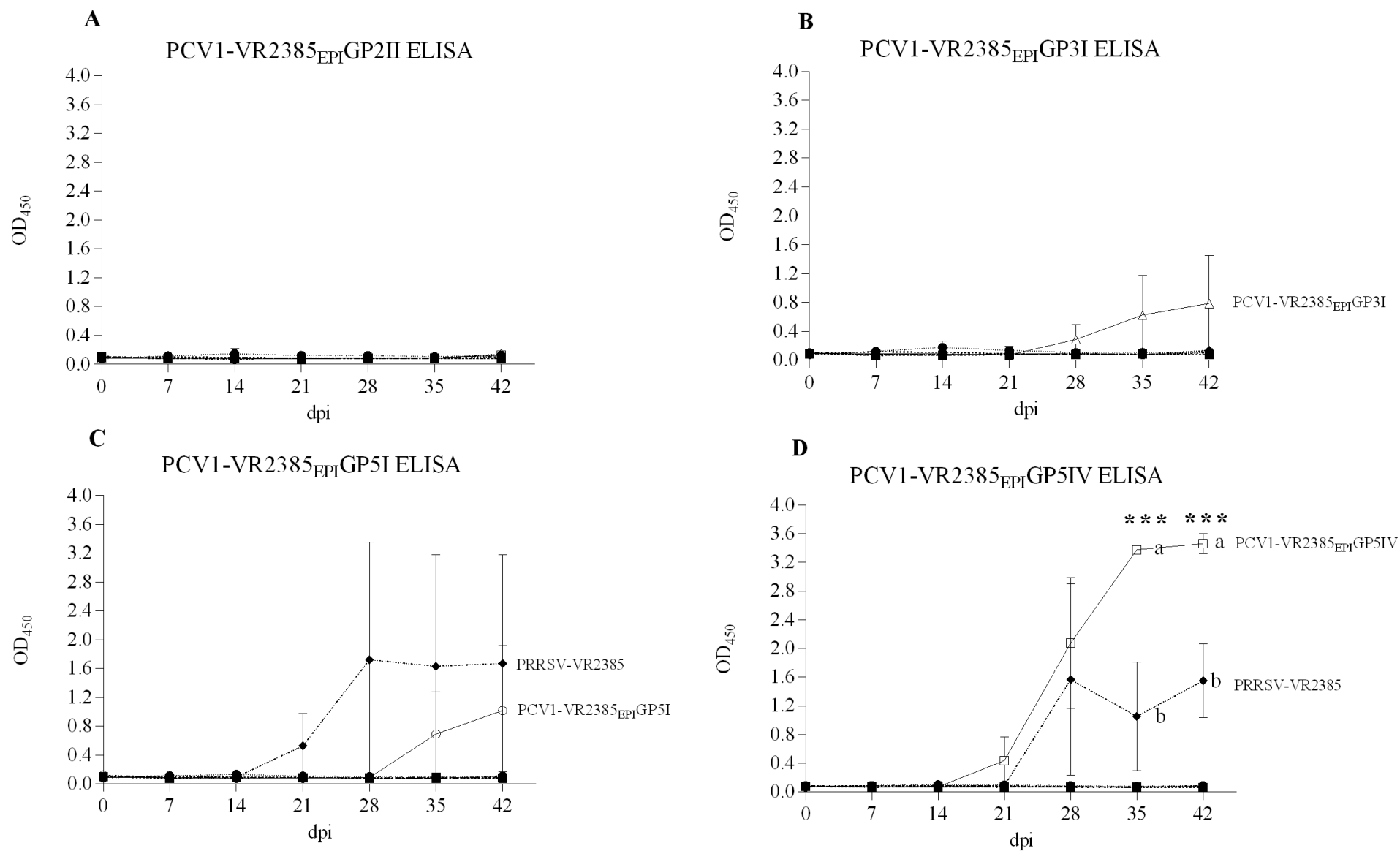


Fig 6

